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In situ immune infrared fluorescent staining for detection and quantitation of bluetongue virus in *Culicoides* insect cell culture

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Bluetongue virus (BTV) is transmitted to sheep, cattle and other ruminants by *Culicoides* spp. of biting midges. Cell lines have been developed from *Culicoides sonorensis*; however, techniques to detect and quantitate viable virus directly in these insect cells are lacking. *In situ* immune infrared fluorescent staining techniques were developed to visualize and quantitate BTV infection in *Culicoides* cell culture by both an endpoint titration and an agarose overlay fluorescent focus assay. Insect cell cultures infected with BTV were fixed, permeabilized and reacted with virus-specific monoclonal antibody and fluorescent-labeled secondary antibody. Virus replication in the infected cells was visualized and quantitated by measuring fluorescence with an infrared imager. The sensitivity of virus detection in insect cell culture using these techniques was comparable to or better than detection by standard techniques in vertebrate cell culture.
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1. Introduction

Bluetongue virus (BTV) is transmitted to sheep, cattle and other ruminants by *Culicoides* spp. of biting midges (Borden et al., 1971; Matthews, 1982; Price and Hardy, 1954). Cell lines developed from *Culicoides sonorensis* have facilitated studies of virus replication and the role of the insect vector in BTV transmission (McHolland and Mecham, 2003; Mertens et al., 1996; Mortola et al., 2004; Nunamaker et al., 1999; Wechsler et al., 1989, 1991; Xu et al., 1997). Bluetongue virus replicates in the *Culicoides* insect cells; however, since there are minimal cytopathic effects, virus is routinely detected by co-cultivation of infected insect cells or cellular material with susceptible vertebrate cells (McHolland and Mecham, 2003; Mertens et al., 1996; Wechsler et al., 1989). Immunoperoxidase staining has been used to directly visualize virus replication in the insect cells (Wechsler et al., 1991). However, this technique is not easily quantitated and suffers from operator subjectivity, since microscopic observation is required. Direct visualization and titration of Dengue virus in *Aedes albopictus* (C6/36) cells by immune fluorescent staining in microtiter plates has been described (Schoepp and Beaty, 1984). In the present study, an *in situ* immune fluorescent staining technique, in combination with an infrared imaging detection system, was used to directly visualize and quantitate BTV infection in *Culicoides* cell culture.

2. Materials and methods

2.1. Cell lines and viruses

The KC and W3 cell lines, derived from *C. sonorensis* insects and developed at the Arthropod-Borne Animal Diseases Research Laboratory, and Vero Maru (VM) cells were used in this study (McHolland and Mecham, 2003; Wechsler et al., 1991). Insect cells were grown in Schneider's modified drosophila medium with 15% heat inactivated fetal bovine serum (FBS), 30 mg/L L-asparagine, 6 mg/L reduced glutathione, 4.5 U/L bovine insulin, an additional 2 mM L-glutamine and 4 ml/L 7.5% $NaHCO_3$. Vero Maru cells were grown in Medium 199 with Earles salts plus 10% FBS and 2.2 g/L $NaHCO_3$. Bluetongue virus serotype 10 (BTV-10), serotype 11 (BTV-11) and epizootic hemorrhagic disease virus serotype 1 (EHDV-1) were produced in baby hamster kidney (BHK-21) cells. Infected cells were disrupted by sonication, centrifuged to remove cellular debris, and the supernatant saved for infection of cell cultures.

2.2. Endpoint titration

Two-way, twofold dilutions of an initial 1:100 dilution of virus (BTV-11 or EHDV-1) were performed in 96-well microtiter plates (final volume of 100 μl/well). Control wells containing diluent, but no virus, were included in each plate. KC cells ($\sim 1.8 \times 10^6$ ml^{-1}) or VM cells ($\sim 7 \times 10^5$ ml^{-1}) (100 μl/well) were then added to each well, and the plates incubated at 34 °C. Schneider's medium with 5%

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heat inactivated FBS was used as diluent and maintenance medium for the KC cells. Medium 199 with 2% FBS was used as diluent and maintenance medium for the VM cells.

KC cells were removed from the 34 °C incubator at either 7 or 10 days after infection (DAI) and the media removed. The cells were fixed with 100 μl/well of 80% acetone/20% deionized water at room temperature for 10 min, after which the acetone solution was removed and the plates allowed to air dry for 10 min at room temperature. The plates were then held at −80 °C overnight. The next day, cell permeability was increased by adding 100 μl/well of 0.01 M phosphate buffered saline (PBS) containing 0.1% Triton-X 100 (PBS/Triton) for 10 min at room temperature. The PBS/Triton was removed and the cells washed three times with PBS containing 0.1% Tween-20 (PBS/Tween). Odyssey Blocking Buffer (LiCor Biosciences, Lincoln, NB) (150 μl/well) was added to the plates for 60 min at room temperature. The blocking buffer was removed and 50 μl/well of monoclonal antibody (1AA4.E4), specific for the BTV, VP7 structural protein (Mecham et al., 1990), diluted 1:200 in Odyssey Blocking Buffer, was then added and the plate incubated at room temperature for 2 h with shaking. The monoclonal antibody solution was removed and the wells washed three times with PBS/Tween using an automated plate washer. IR Dye 800 goat anti-mouse secondary antibody (LiCor Biosciences) (50 μl/well), diluted 1:800 in Odyssey Blocking Buffer, was then added and the plates incubated in the dark on a shaker for 1 h at room temperature. The secondary antibody solution was removed and the wells of the plate washed three times with PBS/Tween. Immune fluorescence was detected and documented with an Odyssey Infrared Imaging System (LiCor Biosciences) (800 nm, intensity 6, focus offset 3.0, contrast 50, and brightness 50). Wells were scored positive, if they exhibited at least 25% of the fluorescent intensity observed in the wells containing the initial virus dilution, and the results used to calculate 50% endpoint ($TCID_{50}$) virus titers (Reed and Muench, 1938).

Vero Maru cells were removed from the incubator 7 DAI with the same virus stocks used to infect the KC cells. The cell culture supernatant was removed and the cells were fixed and stained for cytopathology with 10% PBS buffered formalin and 0.1% crystal violet (Mecham, 1993). Wells were scored positive or negative for cytopathology by visual examination to calculate $TCID_{50}$ virus titers. KC cells infected with virus for 7 days were also fixed and stained for the detection of cytopathology.

2.3. Fluorescent focus assay in insect cells

Twelve well cell culture plates were seeded with 1.25 ml/well of W3 cells at a 1:5 split ratio in Schneider's growth medium and incubated at 34 °C for 3 days. After that time, the medium was removed and the cell monolayer washed once with 1 ml/well of DMEM and once with Dulbecco's PBS + 0.1% BSA (D + B), after which 50 μl of D + B was added to the wells. Virus (BTV-10), diluted in serum-free DMEM, was added to duplicate wells (50 μl/well). Duplicate control wells received only DMEM. The cells were then incubated for 1 h at 34 °C with occasional rocking. Following incubation, the virus/D + B mixture was removed and 1.25 ml of overlay medium (0.5% agarose in Schnieders maintenance medium) was added to each well. After the overlay solidified, the plates were incubated at 34 °C for 4 days. Following incubation, the cells were fixed by the addition of 1 ml/well 80% acetone for 10 min at room temperature. The acetone was discarded and the agarose overlay removed with a gentle stream of PBS. The cell monolayer was fixed for an additional 10 min at room temperature with acetone. Subsequently, the acetone was removed and the plates were air dried for 15 min at room temperature and stored at −80 °C until stained. To stain, the plates were removed from the freezer and allowed to thaw at room temperature. After thawing, PBS/Triton (500 μl/well) was added to the cells for 10 min. The cells were washed three times with 500 μl/well washing buffer (PBS/Tween) for 5 min/wash, after which Odyssey Blocking Buffer (1 ml/well) was added to the cells and the plates incubated at room temperature on a shaker for 1 h. This was followed by the addition of 300 μl/well of primary antibody (mouse monoclonal antibody, 1AA4.E4) diluted in Odyssey Blocking Buffer for 1 h at room temperature with shaking. The monoclonal antibody solution was discarded and the cells washed four times with 500 μl/well of PBS/Tween (5 min/wash). Secondary antibody (IR Dye 800 goat anti-mouse diluted 1:800 in Odyssey Blocking Buffer) was added to the cells (300 μl/well) and the plates incubated for 1 h at room temperature in the dark on the shaker. The secondary antibody solution was then discarded and the cells washed four times with 500 μl/well PBS/Tween in the dark with shaking (5 min/wash). Immune fluorescent foci (FF) were detected and documented with the Odyssey Infrared Imaging System (800 nm, intensity 6, focus offset 3.0, contrast 50 and brightness 50) and the virus titer was calculated as FF/ml.

2.4. Standard plaque assay

Twelve well cell culture plates were seeded with 1 ml/well VM cells (5×10^5 cells/ml) and incubated at 34 °C. The following day, the cells were infected with BTV-10 as previously described for the fluorescent focus assay; with the exception that the cells were overlaid with 0.5% agarose in Medium 199 with 10% heat inactivated FBS. After incubation at 34 °C for 7 days, the cells were fixed with 10% phosphate buffered formalin (1 ml/well) for 1 h at room temperature. The formalin and agarose were removed with a stream of water and the cells stained with 0.1% crystal violet in formalin (1 ml/well) for 1 h at room temperature. The stain was discarded, the wells rinsed with tap water and allowed to air dry at room temperature, after which visible plaques were counted and the virus titer was calculated as PFU/ml.

3. Results

3.1. Endpoint titrations

Endpoint titrations of stock virus in both KC and VM cells are shown in Fig. 1. Staining with crystal violet allowed visualization of gross cytopathology in infected VM cells (Fig. 1A, bottom panel). However, standard crystal violet staining of the KC insect cell line could not be used to determine virus titer, since gross cytopathology was not detectable in these cells following infection with BTV (Fig. 1A, top panel). An *in situ* immune infrared fluorescent staining technique was developed that detected the presence of BTV, VP7 structural protein in these cells (Fig. 1B). The calculated titer of the stock virus in the KC cells 7 DAI and staining was 4.6×10^5 $TCID_{50}$/ml and was higher than the calculated titer after infection and staining of VM cells (8.9×10^4 $TCID_{50}$/ml). If the KC cells were examined at 10 DAI, the calculated titer of the stock virus increased to 5.5×10^5 $TCID_{50}$/ml. The immune fluorescent staining technique was specific for BTV and did not detect the related EHDV, even though cytopathology was clearly evident in VM cells following infection with this virus (Fig. 2).

3.2. Fluorescent focus and plaque assays

A fluorescent focus assay was also developed to detect BTV specific antigen in insect cell lines that do not exhibit visible cytopathic effects. In the W3 *Culicoides* cell line (Fig. 3, bottom panel), the fluorescent foci observed after infection were discrete and easily countable at 4 DAI. Longer incubation periods resulted in coalescence of foci. Nonspecific background fluorescence was minimal

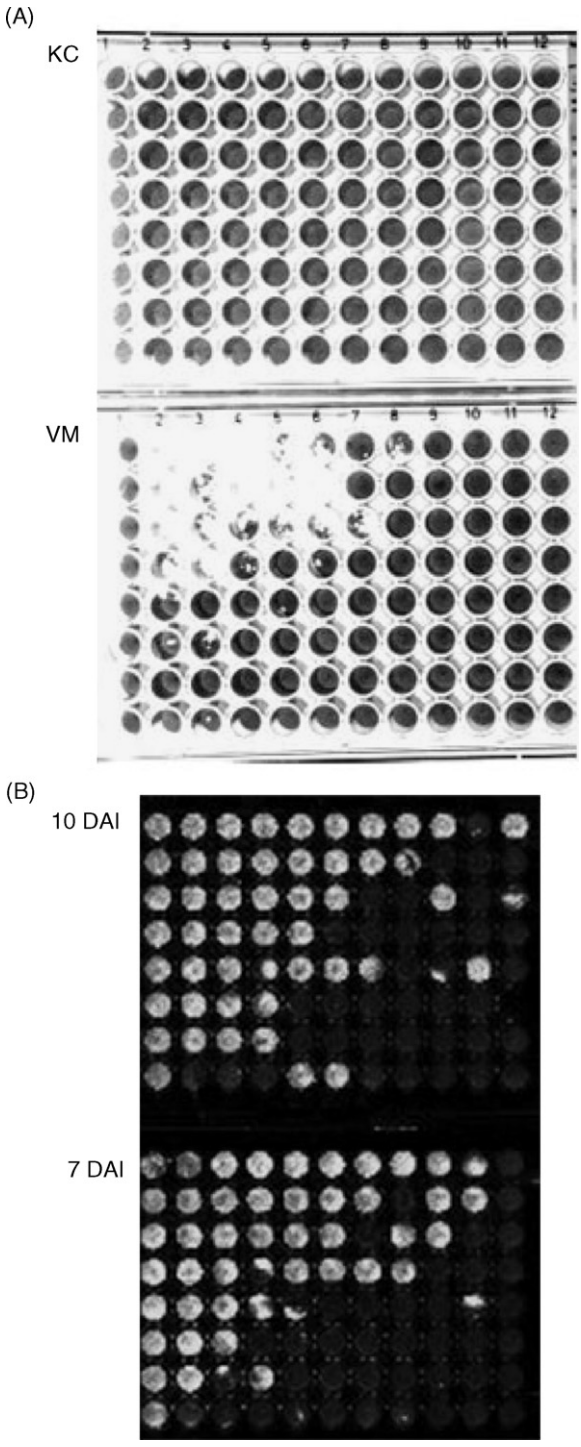

Fig. 1. Endpoint titration of BTV. Insect cells (KC) and Vero Maru cells (VM) were set in 96-well culture plates and infected with BTV-11 diluted in a two-way, twofold (across and down the plate) pattern starting with a 1:100 dilution of stock virus. (A) At 7 days after infection (DAI), both KC and VM cells were fixed with 10% formalin and then stained for gross cytopathology with crystal violet. (B) At 7 and 10 DAI, KC cells were processed for immune fluorescent detection of BTV using the LiCor Odyssey Infrared Imaging System.

when monoclonal antibody, 1AA4.E4, was used as the primary antibody and did not interfere with visualization of the fluorescent foci. The titer of the BTV-10 stock virus determined by this assay was 1.9×10^5 FF/ml. Titration of the same virus in VM cells by a traditional agarose overlay and crystal violet staining procedure (Fig. 3, top panel) required 7 days to obtain discrete, countable plaques.

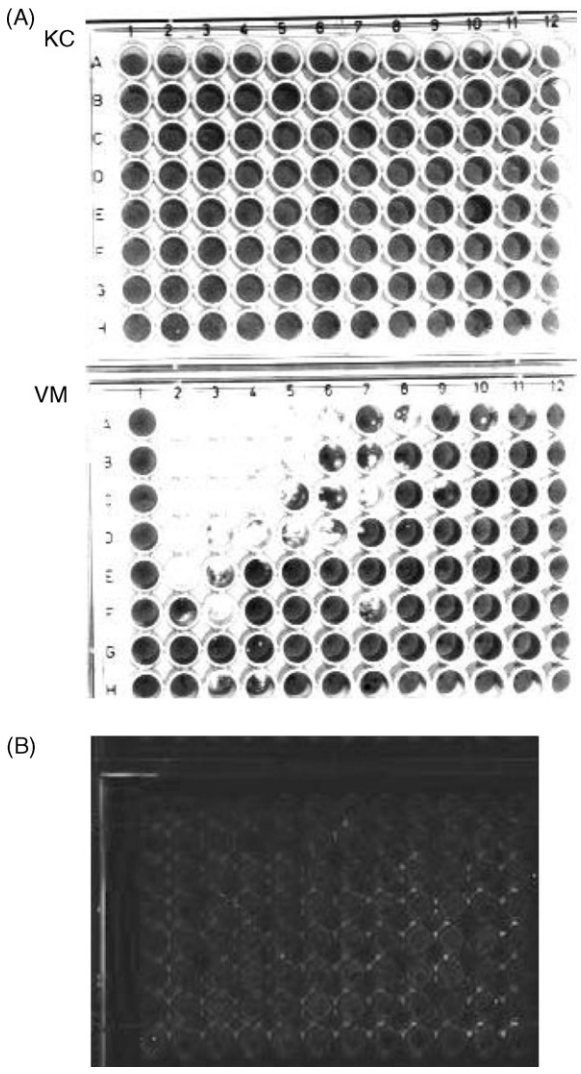

Fig. 2. Endpoint titration of EHDV. Insect cells (KC) and Vero Maru cells (VM) were set in 96-well culture plates and infected with EHDV-1 diluted in a two-way, twofold (across and down the plate) pattern starting with a 1:100 dilution of stock virus. (A) At 7 days after infection (DAI), both KC and VM cells were fixed with 10% formalin and then stained for gross cytopathology with crystal violet. (B) At 10 DAI, KC cells were processed for immune fluorescent detection of BTV using the LiCor Odyssey Infrared Imaging System.

The stock virus titer determined by this conventional plaque assay was 1.9×10^5 PFU/ml.

4. Discussion

In situ immune infrared fluorescent staining techniques were developed that detect BTV infection directly in *Culicoides* insect cell cultures. These techniques were used to quantitate viable virus in the absence of visible cytopathic effects. Titers were comparable to or higher than those obtained in mammalian cell culture (VM) using traditional endpoint titration and plaque assay. The ability to quantitate non-cytopathic virus replication directly in the *Culicoides* cell lines will improve the reliability and accuracy of assays performed with these cells; and these techniques could be modified to accommodate a variety of viruses by substituting the appropriate primary monoclonal antibody.

Two insect cell lines were used to develop the *in situ* immune fluorescent detection techniques. The KC cell line was derived from colonized *C. sonorensis*, while the W3 cell line was derived from field collected *C. sonorensis* (McHolland and Mecham, 2003; Wechsler et

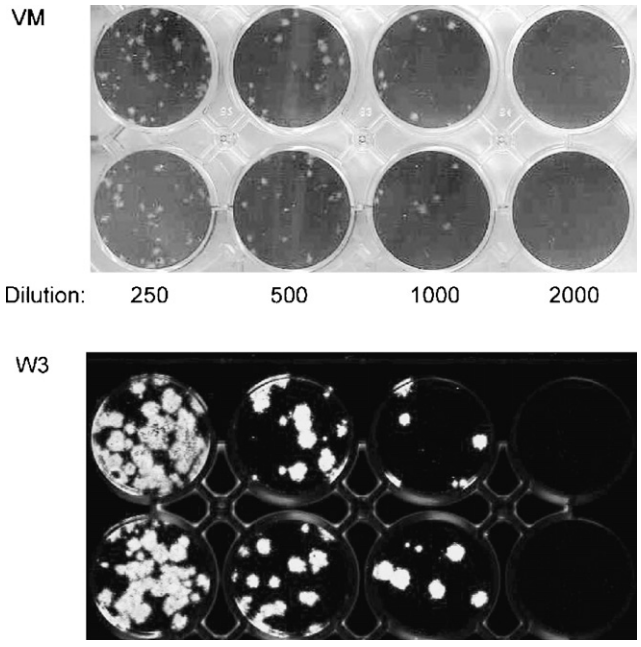

Fig. 3. Plaque assay and immune fluorescent focus assay for the detection of BTV. Vero Maru (VM) and insect cells (W3) were set in 12-well culture plates and infected with serial dilutions of BTV-10, overlaid with agarose and incubated at 34 °C. At 7 days after infection (DAI) the VM cells were fixed with formalin and stained with crystal violet to detect plaque forming units (top panel). At 4 DAI, the W3 cells were processed for immune fluorescent detection of fluorescent foci using the LiCor Odyssey Infrared Imaging System.

al., 1991). Both cell lines were evaluated in developing the two new assays described in this paper. The W3 cells performed better in the fluorescent focus assay. Fewer cells were lost during the staining process, thus improving reproducibility, and less time was required to obtain readable results. Infected KC cells required at least 7 days of incubation before quite small fluorescent foci were observed. The optimum incubation period for the development of fluorescent foci in the W3 cell line was 4 days. McHolland and Mecham (2003) reported that BTV replicated to equivalent titers in both the KC and W3 cell lines, but that the virus titer peaked in 2 days in the W3 cells, while maximum titers were not observed in the KC cells until 7 DAI. Bluetongue virus, VP7 antigen, was also detected earlier in the W3 cells by immune precipitation (unpublished data).

In the endpoint titration assay, however, more consistent reproducible results were obtained using the KC cell line and the methods described. With the W3 cells, there was significant loss of fluorescent staining in wells infected with higher concentrations of virus. Under normal growth conditions, the W3 cells are more adherent than the KC cells. However, after infection of the W3 cells with high titered BTV they often become more rounded. This morphological change could cause the cells to be less adherent. As a result, consistent reproducible results would be more difficult to obtain in wells with higher concentrations of BTV in the endpoint titration assay because more cells would be lost during postinfection processing.

An antigen capture ELISA for titration of BTV in *Culicoides* cell lines has been described (Mecham, 2006). However, that assay requires release of virus by the disruption of the infected cells. The *in situ* assays described in this study detect virus directly in the infected cells. Conventional plaque assay, which detects gross, visible cytopathology in a cell culture monolayer, has been described for BTV using different vertebrate cell types (Howell et al., 1967; Wechsler and McHolland, 1988). However, such assays for BTV using insect cell culture have not been possible because of minimal cytopathology following infection. The fluorescent focus assay described in this study is the first report of a "plaque" type assay for BTV in *Culicoides* cell culture. Since the insect cell lines used in this study were derived from the vector of BTV, as well as related *orbiviruses*, the assays described have potential for numerous research and diagnostic applications.

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